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# Is *Neisseria gonorrhoeae* Initiating a Future Era of Untreatable Gonorrhea?: Detailed Characterization of the First Strain with High-Level Resistance to Ceftriaxone▽†

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**Recently, the first *Neisseria gonorrhoeae* strain (H041) that is highly resistant to the extended-spectrum cephalosporin (ESC) ceftriaxone, the last remaining option for empirical first-line treatment, was isolated. We performed a detailed characterization of H041, phenotypically and genetically, to confirm the finding, examine its antimicrobial resistance (AMR), and elucidate the resistance mechanisms. H041 was examined using seven species-confirmatory tests, antibiograms (30 antimicrobials), *porB* sequencing, *N. gonorrhoeae* multiantigen sequence typing (NG-MAST), multilocus sequence typing (MLST), and sequencing of ESC resistance determinants (*penA*, *mtrR*, *penB*, *ponA*, and *pilQ*). Transformation, using appropriate recipient strains, was performed to confirm the ESC resistance determinants. H041 was assigned to serovar Bpyust, MLST sequence type (ST) ST7363, and the new NG-MAST ST4220. H041 proved highly resistant to ceftriaxone (2 to 4 μg/ml, which is 4- to 8-fold higher than any previously described isolate) and all other cephalosporins, as well as most other antimicrobials tested. A new *penA* mosaic allele caused the ceftriaxone resistance. In conclusion, *N. gonorrhoeae* has now shown its ability to also develop ceftriaxone resistance. Although the biological fitness of ceftriaxone resistance in *N. gonorrhoeae* remains unknown, *N. gonorrhoeae* may soon become a true superbug, causing untreatable gonorrhea. A reduction in the global gonorrhea burden by enhanced disease control activities, combined with wider strategies for general AMR control and enhanced understanding of the mechanisms of emergence and spread of AMR, which need to be monitored globally, and public health response plans for global (and national) perspectives are important. Ultimately, the development of new drugs for efficacious gonorrhea treatment is necessary.**

Gonorrhea, caused by *Neisseria gonorrhoeae* (gonococcus), is the second-most-prevalent bacterial sexually transmitted infection globally. The disease is associated with high morbidity and socioeconomic consequences and remains a public health problem worldwide (36, 46; G. Schmid, presented at WHO/CDC symposium: Congenital syphilis and the 2005 WHO estimates of STI incidence and prevalence: using the second to help eliminate the first, 18th International Society for Sexually Transmitted Disease Research conference [ISSTDR], 28 June to 1 July 2009, London, United Kingdom). In the absence of a vaccine, appropriate diagnostics and antimicrobial therapy are the key elements for reduction and control of gonorrhea and the development of associated severe complications and sequelae, as well as further transmission of the infection (34, 36).

The treatment options, however, have diminished rapidly because of the emergence and worldwide spread of antimicrobial resistance (AMR) to all drugs previously used or considered first line, i.e., penicillins, narrow-spectrum cephalosporins, tetracyclines, macrolides, and fluoroquinolones. Furthermore, rapid emergence of resistance to spectinomycin was observed when it was widely used for treatment in the past (4), and this antimicrobial is not suitable for treatment of pharyngeal gonorrhea, nor is it currently available in many countries (3, 15, 36). Accordingly, spectinomycin is not a promising candidate for first-line empirical treatment of gonorrhea. Worryingly, in recent years, susceptibility to the currently recommended first-line antimicrobials, the extended-spectrum cephalosporins (ESCs), i.e., ceftriaxone (injectable) and cefixime (oral), has also decreased globally (3, 15, 17, 36). Furthermore, for several years, cefixime treatment failures have been recognized in Japan (9, 36, 47), where cefixime was already excluded from treatment guidelines in 2006 (36). More recently, failures have also been verified in Europe (40). However, despite the fact that susceptibility to ceftriaxone (the last remaining option for empirical first-line treatment) is decreasing globally, *in vitro* and clinical (resulting in treatment failure of urogenital gonorrhea) resistance has been lacking (3, 15, 17, 36).

Recently, the first high-level ceftriaxone-resistant gonococcal strain (H041) was isolated from the pharynx of a female commercial sex worker in Kyoto, Japan (23). H041 displayed a MIC of ceftriaxone of 2 μg/ml. This is a very high level of resistance and, previously, only one isolate having an MIC of >0.25 μg/ml (MIC = 0.5 μg/ml) (33) has been reported world-

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wide. Unfortunately, it was not possible to definitively verify that H041 caused a treatment failure because a posttreatment isolate was not available; however, treatment failure seems likely (see Discussion). Furthermore, H041 belongs to multilocus sequence typing (MLST) sequence type (ST) ST7363 and is closely related to the successful gonococcal cefixime-resistant subclones of ST7363 (23), which are prevalent in Japan (24) and now are also being transmitted in Europe. Accordingly, H041 may be a subclone of the MLST ST7363 cefixime-resistant strains that has acquired additional resistance determinant(s), resulting in high-level ceftriaxone resistance. Historically, gonococcal AMR has mostly emerged in the World Health Organization (WHO) Western Pacific Region (WPR) and, in particular, in Japan. This resistance has spread rapidly, via sex tourists, long-distance truck drivers, and forced migration in the WHO WPR, to the Pacific Rim countries, including the United States, Southeast and Central Asia, Europe, and globally (36). The spread of ceftriaxone-resistant gonococcal strains worldwide will probably follow the same pattern. Consequently, it is crucial to examine in detail, including elucidation of the mechanisms causing the ceftriaxone resistance, the first high-level ceftriaxone-resistant gonococcal strain worldwide, recently isolated in Japan (H041) (23).

The most common mechanism in gonococci for decreased ESC susceptibility is alteration of the *penA* gene, i.e., the acquisition of a *penA* mosaic allele or alterations of amino acid A501 in the encoded penicillin-binding protein 2 (PBP2) (1, 3, 11, 14–18, 21, 23, 24, 27, 28, 32, 33, 36, 38, 40, 41, 44, 50). Mutations in the promoter and/or coding sequence of the repressor gene *mtrR* cause an overexpression of the MtrCDE efflux pump system, which further decreases ESC susceptibility (3, 11, 12, 16–18, 30, 36, 38, 43, 48, 50). Furthermore, specific *porB1b* mutations that alter amino acid G101 and A102 in the PorB1b porin (the *penB* resistance determinant) result in additional decreases in ESC susceptibility (3, 11, 16–18, 25, 26, 30, 36, 38, 50). Nevertheless, based on the relatively few studies of gonococcal isolates with decreased ESC susceptibility, polymorphisms in *ponA* (encoding PBP1) and *pilQ* (encoding the pore-forming secretin PilQ protein in the type IV pili), which both can be involved in high-level penicillin resistance, do not seem to substantially enhance the MICs of ESCs (11, 29, 45, 49, 50). At least one unknown resistance determinant exists (11, 18, 38, 50).

The aims of this study were to perform a detailed characterization, phenotypic and genetic, of the first identified high-level ceftriaxone-resistant *N. gonorrhoeae* strain (H041) worldwide in order to confirm this finding, to thoroughly examine its antimicrobial resistance, and to elucidate the ESC resistance mechanisms.

## MATERIALS AND METHODS

***Neisseria gonorrhoeae* strains.** The high-level ceftriaxone-resistant strain H041 (23) and gonococcal strains ($n = 9$) selected for transformation assays to verify the resistance mechanisms, i.e., five clinical strains and four of the eight 2008 WHO *N. gonorrhoeae* reference strains (41), were included in this study. For gonococcal species verification of H041, morphology on selective culture medium, catalase and oxidase tests, microscopy after Gram staining, and seven species confirmatory tests (sugar utilization, HN-20 Rapid system identification [ID] test [Nissui, Tokyo, Japan], PhadeBact GC monoclonal test [Bactus AB, Solna, Sweden], PhadeBact GC monoclonal serovar test [Bactus AB, Solna, Sweden], MicroTrak *N. gonorrhoeae* culture confirmation test [Trinity Biotech, Wicklow, Ireland], *porA* pseudogene PCR [13], and dual-target PCR [*porA* and *opa*] [10]) were used. All strains were grown on GC culture medium as previously described (42).

**Antimicrobial susceptibility testing.** Ceftriaxone MIC determination was performed using the Etest method (AB bioMérieux, Solna, Sweden) according to the manufacturer's instructions. The ceftriaxone MIC of H041 was also confirmed using the agar dilution method according to the Clinical and Laboratory Standards Institute (CLSI) standards (8). Finally, H041 was examined for its MICs of 29 additional antimicrobials (using the Etest method) and tested with the calibrated dichotomous sensitivity (CDS) disc diffusion method (5, 35), which is used in resistance surveillance of *N. gonorrhoeae* in many countries in the WHO WPR (six antimicrobials) (Table 1). β-Lactamase production was tested using nitrocefin discs. The 2008 WHO *N. gonorrhoeae* reference strains (41) were used for quality controls in all antimicrobial susceptibility testing.

**Genetic characterization.** DNA was isolated in a NorDiag Bullet instrument (NorDiag ASA Company, Oslo, Norway) using a Bugs'n Beads STI-*fast* kit (NorDiag ASA Company, Oslo, Norway) according to the manufacturer's instructions. For molecular epidemiological examination, strains were genotyped by means of MLST (24), *porB* gene sequencing, and *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) as described previously (39). PCR and sequencing of resistance determinants, i.e., the *penA*, *mtrR*, *porB1b*, *ponA*, and *pilQ* genes, were performed as described elsewhere (18, 41, 45).

**Sequence alignments and phylogenetic analysis.** Multiple-sequence alignments (nucleotide and amino acid sequences) were performed using BioEdit Sequence Alignment Editor software (version 7.0.9.0). For examination of the evolutionary relationships of H041 with other *penA* mosaic strains displaying decreased ESC susceptibility and circulating worldwide, a phylogenetic analysis using the full-length *porB* sequences in H041 and previously reported *penA* mosaic strains (11) was performed with TREECON (version 1.3b) as previously described (39).

**Transformation assays.** To confirm that the unique *penA* allele in H041 ($penA_{H041}$) caused the high-level resistance to ceftriaxone, the full-length $penA_{H041}$ was PCR amplified and transformed into nine recipient strains as previously described (24). These nine recipient strains displayed different molecular epidemiological sequence types, ceftriaxone MICs, and composition of ESC resistance mechanisms, such as *penA* alleles, the *mtrR* promoter, and *penB* sequences (Table 2). Briefly, the recipients were suspended in GC broth ($1 \times 10^8$ cells/100 μl) and incubated with 0.2 μg of the $penA_{H041}$ PCR product (after purification using a High Pure PCR product purification kit [Roche Diagnostics GmbH, Mannheim, Germany]) for 4 h. Aliquots of 10 μl and 100 μl were inoculated on GC agar with a 4-fold higher ceftriaxone concentration than the MIC of the respective recipient. After incubation, the colonies obtained were subcultured on an antimicrobial-free GC agar plate for single-clone isolation. For confirmation, the transformation assay was performed three times for each recipient.

**Nucleotide sequence accession numbers.** The GenBank/EMBL/DDBJ accession numbers for the two new *penA* alleles reported in this paper are AB546858 and AB608050.

## RESULTS

**Phenotypic characterization of the high-level ceftriaxone-resistant *N. gonorrhoeae* strain H041.** All conventional bacteriological diagnostic tests and the seven species confirmatory assays verified that H041 was a gonococcal strain, which was assigned to serovar Bpyust.

The results of the antimicrobial susceptibility testing using the Etest method (30 antimicrobials) and CDS disc diffusion method (six antimicrobials) are summarized in Table 1. Briefly, H041 was resistant to various antimicrobials, including all β-lactam antimicrobials (with possible exceptions of carbapenems, at least ertapenem and meropenem, and piperacillin-tazobactam, for which no breakpoints are available), fluoroquinolones, macrolides, tetracycline, trimethoprim-sulfamethoxazole, chloramphenicol, and nitrofurantoin. The MICs of all the cephalosporins, including the recommended first-line ESCs, were very high (e.g., 2 to 4 μg/ml of ceftriaxone and 8 μg/ml of cefixime). H041 did not produce any β-lacta-

TABLE 1. MIC using the Etest method and zone sizes with the calibrated dichotomous sensitivity disc diffusion method of *Neisseria gonorrhoeae* H041 to various antimicrobials

| Antimicrobial | Class | MIC Etest result in μg/ml (agar dilution result), interpretation[a,b] | CDS (mm)[c] |
|---|---|---|---|
| Penicillin G | β-Lactams, penicillins | 4, R[a,b] | 0 |
| Ampicillin | | 2 | ND |
| Amdinocillin | | >256 | ND |
| Piperacillin-tazobactam | | 0.25 | ND |
| Aztreonam | β-Lactam, monobactam | 128 | ND |
| Cefuroxime | β-Lactams, cephalosporins | 16, R[a] | ND |
| Cefpodoxime | | 16, R[a] | 3 |
| Ceftazidime | | 16, R[a] | ND |
| Cefotaxime | | 8, R[a,b] | ND |
| Cefixime | | 8 (8), R[a,b] | ND |
| Ceftriaxone | | 4 (2), R[a,b] | 1 |
| Cefepime | | 16, R[a] | ND |
| Ertapenem | β-Lactams, carbapenems | 0.064 | ND |
| Meropenem | | 0.125 | ND |
| Imipenem | | 2 | ND |
| Ciprofloxacin | Fluoroquinolones | >32, R[a,b] | 1 |
| Levofloxacin | | >32 | ND |
| Moxifloxacin | | 6 | ND |
| Azithromycin | Macrolides | 1, R[b] | ND |
| Erythromycin | | 2 | ND |
| Gentamicin | Aminoglycosides | 4 | ND |
| Kanamycin | | 16 | ND |
| Tobramycin | | 6 | ND |
| Spectinomycin | Aminocyclitol | 16, S[a,b] | 9 |
| Tetracycline | Tetracycline | 4, R[b] | 5 |
| Tigecycline | Glycylcycline | 0.5 | ND |
| Trimetoprim-sulfamethoxazole | Folic acid antagonists | 1 | ND |
| Chloramphenicol | — | 4 | ND |
| Nitrofurantoin | — | 4 | ND |
| Rifampin | — | 0.25 | ND |

[a] Antimicrobial susceptibility testing was performed using the Etest method (AB bioMérieux, Solna, Sweden) on all antimicrobials according to the instructions from the manufacturer (results were rounded up to whole MIC dilutions). Furthermore, agar dilution was additionally performed for ceftriaxone and cefixime (in parentheses) according to the method described by the Clinical Laboratory and Standards Institute (CLSI) (8). Where available, interpretative criteria (S, susceptible; I, intermediate; R, resistant) from the Clinical and Laboratory Standards Institute (CLSI) (8) were used.

[b] Interpretative criteria (S, I, R) from the European Committee on Antimicrobial Susceptibility Testing (EUCAST). Available online at: http://eucast.org/ (Accessed 30 April, 2011).

[c] The calibrated dichotomous sensitivity (CDS) disc diffusion method (5, 35) is used for antimicrobial resistance testing in many countries in the World Health Organization (WHO) Western Pacific Region. ND, not determined.

mase. The strain was, however, susceptible to spectinomycin and rifampin. Furthermore, the MICs of aminoglycosides and tigecycline were also relatively low (no breakpoints are available for these antimicrobials). The CDS disc diffusion method also identified H041 as resistant to ceftriaxone, cefpodoxime, penicillin G, tetracycline, and ciprofloxacin but susceptible to spectinomycin (Table 1).

**Genetic characterization and elucidation of the mechanisms causing the high-level ceftriaxone resistance in *N. gonorrhoeae* strain H041.** The molecular epidemiological characterization assigned H041 as MLST ST7363 and as the not-previously described NG-MAST sequence type ST4220 (www.ng-mast.net). A phylogenetic analysis using *porB* sequences showed that H041 is closely related to other *penA* mosaic strains with decreased ESC susceptibility that have been shown to circulate worldwide (Fig. 1).

The sequencing of ESC resistance determinants showed that H041 possessed a unique *penA* mosaic allele ($penA_{H041}$) and the previously described *mtrR*, *penB*, and *ponA1* (L421P) resistance determinants. No new *pilQ* mutations were found. Thus, the only new resistance determinant, which consequently was suspected to cause the high ESC MICs, was $penA_{H041}$ (GenBank accession number AB546858). $penA_{H041}$ was highly similar (i.e., 97.6% nucleotide identity and only 12 PBP2 amino acid differences that clustered in two regions) to the previously described *penA* mosaic allele X that has been correlated with cefixime treatment failures in Japan. Of these 12 amino acids, five were unique compared with any gonococcal PBP2 sequence previously described, but one of these (I486) has been found in *Neisseria meningitidis* and *Neisseria flavescens* (Fig. 2). Accordingly, $penA_{H041}$ contained only four PBP2 amino acid residues that have not been previously reported in any *Neisseria* species; compared to *penA* mosaic X, these consisted of A311V, T316P, A328T, and T484S (Fig. 2).

**Transformation assays confirm that the unique $penA_{H041}$ caused the high-level resistance to ceftriaxone and other extended-spectrum cephalosporins.** Based on their different genotypes, ceftriaxone MICs, and composition of ESC resistance determinants, nine strains were selected as recipients of $penA_{H041}$ in transformation assays (Table 2).

Upon transformation with $penA_{H041}$, the ceftriaxone MICs of the recipients increased to 0.125 to 8 μg/ml, i.e., by 16- to 500-fold. Accordingly, the ceftriaxone MICs of all recipients, with the exception of NG9901 (0.125 μg/ml), increased above

TABLE 2. *Neisseria gonorrhoeae* strains with different ceftriaxone MICs and containing divergent genetic ceftriaxone resistance mechanisms that were used as recipients in transformation of the full-length *penA* gene from H041

| Strain | MLST[a] | NG-MAST[b] | Ceftriaxone MIC (μg/ml)[c] | *penA*[d] (allele) | *mtrR*[e] | *penB*[f] | *ponA*[g] |
|---|---|---|---|---|---|---|---|
| NG9901 | ST7363 | ST240 | <0.002 | *penA* XXXVI (mosaic[h]) | WT | WT | WT |
| WHO F | NEW | ST3303 | <0.002 | *penA* XV (WT) | WT | WT | WT |
| NG9903 | ST7359 | ST4058 | 0.004 | *penA* II (A345a) | WT | WT | WT |
| NG9807 | ST7363 | ST4093 | 0.016 | *penA* II (A345a) | A-del | Yes | L421P |
| WHO M | ST7367 | ST3304 | 0.016 | *penA* II (A345a) | A-del | Yes | L421P |
| WHO K | ST7363 | ST1424 | 0.064 | *penA* X (mosaic) | A-del | Yes | L421P |
| NG0003 | ST7363 | ST4068 | 0.125 | *penA* X (mosaic) | A-del | Yes | L421P |
| 35/02 | ST7363 | ST326 | 0.125 | *penA* XXVIII (mosaic) | A-del | Yes | L421P |
| WHO L | ST1590 | ST1422 | 0.125 | *penA* VII (A501V) | A-del | Yes | L421P |

[a] MLST, multilocus sequencing typing (24).
[b] NG-MAST, *Neisseria gonorrhoeae* multiantigen sequence typing (39).
[c] Etest results were rounded up to whole MIC steps. MICs of <0.002 μg/ml were calculated as 0.001 μg/ml in the MIC ratios in Fig. 3.
[d] The *penA* mosaic allele encodes a mosaic penicillin binding protein 2 (PBP2) that causes a decreased susceptibility to extended-spectrum cephalosporins. Mosaic X has been found in cefixime-resistant *N. gonorrhoeae* isolates in Japan (18, 36, 50).
[e] A-del indicates a characteristic single nucleotide (A) deletion in the inverted repeat of the promoter region of *mtrR* that causes an overexpression of the MtrCDE efflux pump that results in a further decrease in susceptibility to ESCs (18, 36, 50).
[f] "Yes" indicates the presence of the alterations of amino acids 120 and 121 in porin PorB (*penB* alteration) that cause a decreased intake of ESCs and, accordingly, a further decrease in susceptibility to ESCs (18, 36, 50). WT, wild type.
[g] The alteration of amino acid 421 in PBP1 (encoded by *penA*) causes a decreased susceptibility to penicillins (18, 29, 50).
[h] *penA* mosaic allele that has not been previously described and whose sequence has been deposited in GenBank with accession number AB608050.

the resistance breakpoint (>0.25 μg/ml) (8) independent of other resistance determinants. Remarkably, WHO F, which has wild-type alleles of all ESC and penicillin resistance determinants, displayed a ceftriaxone MIC of 0.5 μg/ml after transformation (500-fold MIC increase) (Fig. 3).

All single-clone transformants (derived from all recipient strains) showed *mtrR*, *penB*, and *ponA* sequences identical to those in the recipients. All the single-clone transformants also contained the $penA_{H041}$ allele. In most transformants, the transformed $penA_{H041}$ sequence was identical to the sequence in H041. However, in a few transformants, such as those derived from the WHO F and WHO M strains, some point mutations differed from the $penA_{H041}$ sequence. These were considered to represent spontaneous mutations, mutations in junctions for recombination and/or belonging to the *penA* allele of the recipient. The majority of these mutations were nonsynonymous, and none was located in any segment of the mosaic *penA* allele affecting the ceftriaxone MICs. Consequently, the transformation experiments confirmed that $penA_{H041}$ was the cause of the high-level ceftriaxone resistance.

## DISCUSSION

The present study describes the detailed phenotypic and genetic confirmation and characterization, including elucidation of the resistance mechanisms, of the first identified *N. gonorrhoeae* strain (H041) displaying high-level resistance to ceftriaxone worldwide. H041 was isolated from a female commercial sex worker in Japan (23), and the ceftriaxone MIC of H041 was 4- to 8-fold higher than any previously observed. Ceftriaxone is also the last remaining option for empirical first-line treatment of gonorrhea. Accordingly, *N. gonorrhoeae* has now shown its ability to develop resistance to ceftriaxone also and, although the biological fitness of ceftriaxone resistance in *N. gonorrhoeae* remains unknown, the gonococcus may become a true superbug that initiates a future era of untreatable gonorrhea.

Although a posttreatment isolate was unavailable (only one specimen positive with SDA [ProbeTec ET; Becton-Dickinson], sampled 2 weeks after treatment) to definitively verify treatment failure using 1 g ceftriaxone intravenously (23), it seems likely that this was the first gonorrhea clinical failure caused solely by high resistance of the bacteria to ceftriaxone, based on the posttreatment positive-SDA sample (all residual gonococcal DNA is expected to be eliminated before 2 weeks posttreatment) (2), the very high ceftriaxone MIC, and all available data regarding pharmacodynamic parameters for ESCs. Thus, according to Monte Carlo simulations, the 1 g ceftriaxone intravenously that was used for treatment (in full concordance with treatment recommendations for urogenital and pharyngeal gonorrhea in the Japanese treatment guidelines) results in median times of free ceftriaxone above the MIC ($fT_{>MIC}$) of only 6.0 h (0 to 20.3 h) and 0 h (0 to 5.6 h) for the detected MICs of 2 μg/ml (agar dilution method) and 4 μg/ml (Etest method), respectively (6). Accordingly, using 1 g ceftriaxone for treatment, the ceftriaxone MIC of H041 will make the strain escape eradication in most (if not all) patients. Furthermore, this was a case of pharyngeal gonorrhea, which is substantially harder to treat than urogenital gonorrhea (3, 36), and the infection probably resolved spontaneously within 3 months. Nevertheless, despite the fact that a clinical history was recorded, re-reinfection cannot be completely excluded, especially as the patient was a commercial sex worker.

The resistance determinants causing the high ESC MICs in H041 were also elucidated. The unique $penA_{H041}$ mosaic allele was found to be responsible; upon transformation of $penA_{H041}$ into recipients with different ESC MICs and resistance mechanisms, their ceftriaxone MICs increased to 0.125 to 8 μg/ml, i.e., by 16-fold to 500-fold. Nevertheless, additional resistance determinants, especially *mtrR* and *penB* (and "factor X," i.e., the still unidentified determinant), were needed to reach the same level of ceftriaxone MIC as H041, a synergy that was previously reported (18, 36, 50). Factor X was not transformable using the H041 genome (data not shown), which has also

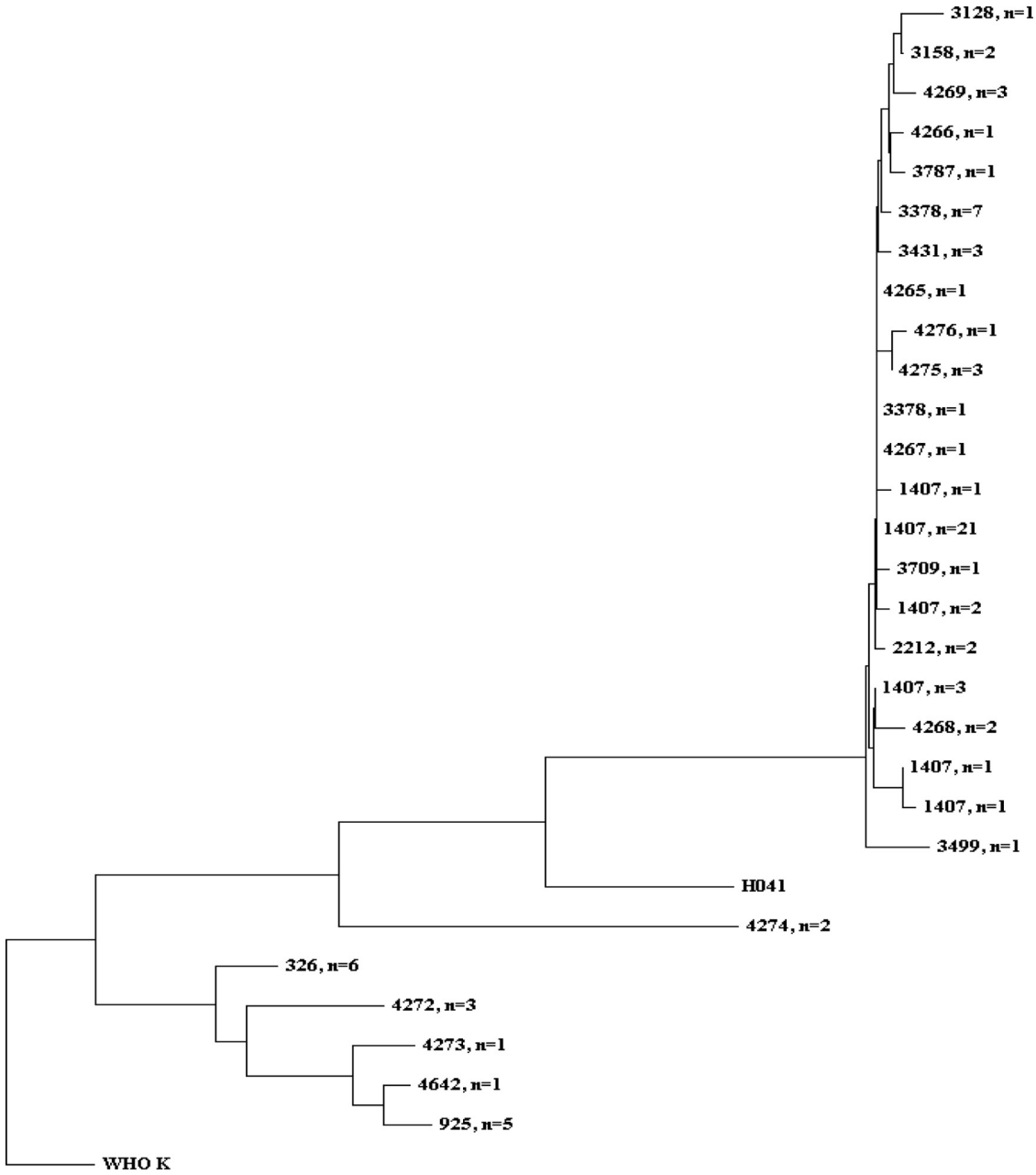


FIG. 1. Phylogenetic tree describing the evolutionary relationships of full-length *porB* gene sequences of the high-level ceftriaxone-resistant *Neisseria gonorrhoeae* strain H041 compared with those of previously published *N. gonorrhoeae penA* mosaic isolates (11). The 2008 WHO K reference strain (41), containing a *penA* mosaic allele X and cultured in Japan in 2001, was used to root the tree. The *N. gonorrhoeae* multiantigen sequence typing (NG-MAST) sequence type (ST) and number of isolates are indicated.

previously been described using other gonococcal genomes (18, 50). $penA_{H041}$ is highly similar to the previously described *penA* mosaic allele X (causing ceftriaxone MICs of only 0.064 to 0.125) (Table 2), which has been correlated with cefixime treatment failures in Japan, having only 12 PBP2 amino acid differences clustering in two regions. Of these 12 amino acids, only four have not been previously reported in any *Neisseria* species; compared with *penA* mosaic X, these consist of A311V, T316P, A328T (in region A), and T484S (in region B) (Fig. 2). It was also confirmed that transformation of only the $penA_{H041}$ region A into the recipients caused, for most, as high a ceftriaxone MIC as transforming the full-length $penA_{H041}$ (data not shown). Although further confirmatory studies are needed, it is highly probable that A311V and T316S are the alterations causing the high ceftriaxone resistance, i.e., due to the proximity to the β-lactam active site in PBP2. Despite this fact, the MICs of some β-lactam antimicrobials, such as penicillins (especially piperacillin-tazobactam) and carbapenems (particularly ertapenem and meropenem), were surprisingly low. $penA_{H041}$ could also easily be transformed to other gono-

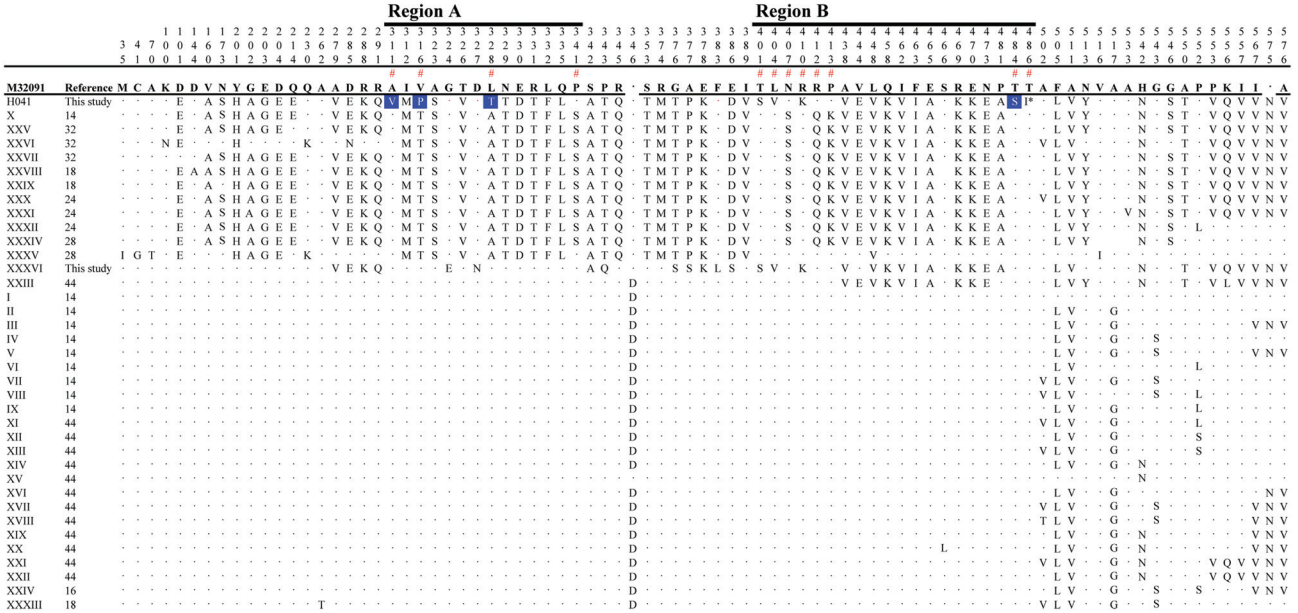


FIG. 2. A schematic figure describing all reported penicillin-binding protein 2 (PBP2) amino acid sequences in *Neisseria gonorrhoeae*, which are aligned to the wild-type PBP2 sequence M32091. All amino acid alterations in the different PBP2 sequences are illustrated with a single capital letter. The amino acids in PBP2 of H041 differing from PBP2 mosaic X ($n = 12$) are indicated (#). The four amino acid residues in the highly ceftriaxone-resistant *N. gonorrhoeae* strain H041 not previously observed in any *Neisseria* species and which explained the ceftriaxone resistance are shown by white letters on a blue ground. The amino acid residue marked with an asterisk has previously been found in *N. meningitidis* (unpublished) and *N. flavescens* (GenBank accession number M26645).

coccal strains in cocultivation experiments (data not shown) performed as previously described (24), which shows that this ceftriaxone resistance can rapidly spread within the *N. gonorrhoeae* population.

H041 seems to represent a subclone of the previously described MLST ST7363 cefixime-resistant *N. gonorrhoeae* circulating in Japan (23, 24). This clone has caused treatment failures using oral ESCs, has successfully spread worldwide, and now seems to have evolved further and developed resistance to ceftriaxone as well. The fear is that this ceftriaxone-resistant subclone will now spread in Japan, to WHO WPR countries, to Pacific Rim countries, and globally, which has been the scenario for emergence and worldwide spread of most gonococcal AMR. Based on previous experience (e.g., for fluoroquinolones), AMR can be widely disseminated internationally only 1 or 2 decades after the first emergence of AMR in WHO WPR (34, 36). The finding of this single high-level ceftriaxone-resistant gonococcal strain is important, especially because it was identified in a female commercial sex worker belonging to a high-risk, frequently transmitting population and because no national gonococcal antimicrobial resistance surveillance programs (including no sentinel sites for identification of gonorrhea treatment failures) are active in Japan. Accordingly, the strain should have excellent opportunities for a rapid spread. An enhanced but still limited gonococcal antimicrobial resistance surveillance in Kyoto, Japan, was initiated after the finding of H041; however, any secondary spread of H041 (or additional treatment failures) has yet not been identified. Despite the suboptimal Japanese surveillance systems, this fact may indicate that H041 has a lower biological fitness that results in limited further spread. Accordingly, the biological fitness of H041, compared to that of its wild type lacking $penA_{H041}$ that causes the ceftriaxone resistance, would be valuable to examine in a well-designed study, i.e., investigating quantitatively the fitness *in vitro* (different culture media, solid and liquid based) and also in an appropriate animal model, i.e., *in vivo*.

Nevertheless, *N. gonorrhoeae* has now shown its ability to develop resistance to ceftriaxone also, in which case gonorrhea may become untreatable in certain circumstances; although the biological fitness of H041 remains unknown, a serious public health problem seems to be approaching. To at least limit the spread of ESC (cefixime and ceftriaxone) resistance, timely and decisive multidisciplinary and multicomponent public health actions are essential not only in Japan but also globally. A recent expert review described WHO initiatives and approaches to AMR containment and how to meet public health challenges of untreatable gonorrhea (36). Nevertheless, to succeed with any AMR containment, enhanced gonorrhea control activities are needed to reduce the burden of infection (36). Furthermore, it is crucial to explore options, in industrialized settings as well as in less-resourced settings, for future treatment of ESC-resistant gonorrhea. This includes exploration of optimized dose regimens of presently used antimicrobials, new antimicrobials (or rediscovery of old drugs, such as gentamicin, ertapenem, and perhaps, piperacillin-tazobactam in emergent situations of ESC-resistant *N. gonorrhoeae*) or other substances, and combination therapy (6, 7, 19, 20, 22, 31, 36, 37; M. Unemo and J. Tapsall, unpublished data).

In conclusion, the first high-level ceftriaxone-resistant *N. gonorrhoeae* strain has now been characterized in detail, including an elucidation of its resistance mechanisms. Accordingly, *N. gonorrhoeae* has now shown its ability to develop

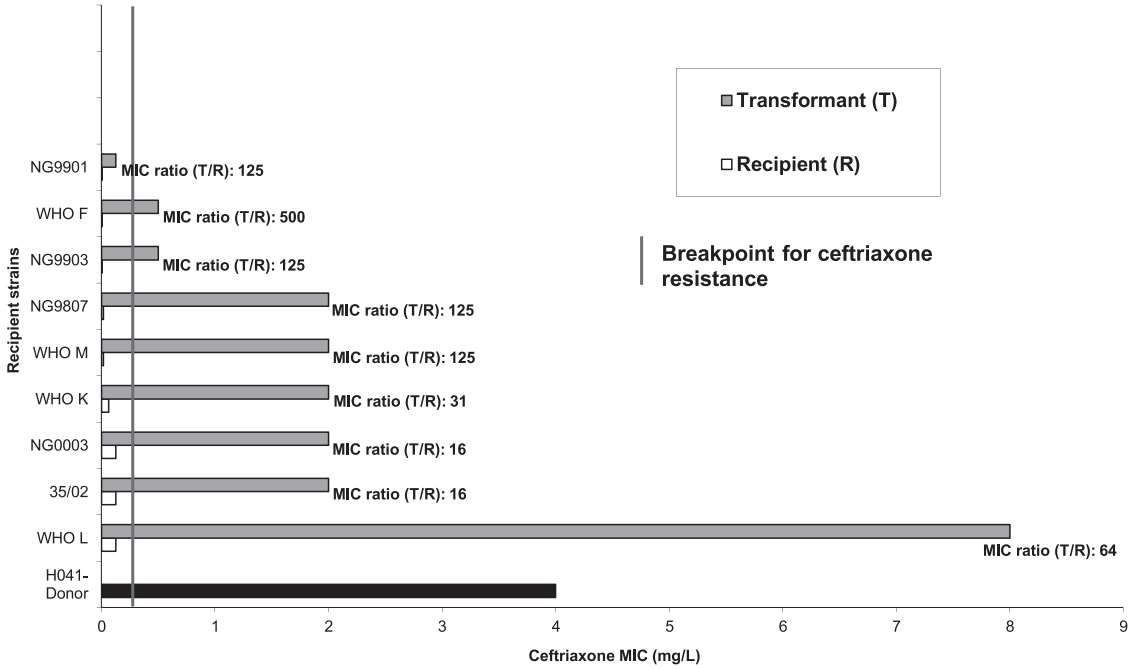


FIG. 3. Transformation of the full-length *penA* allele ($penA_{H041}$) from the high-level ceftriaxone-resistant *Neisseria gonorrhoeae* strain H041 (Donor) into *N. gonorrhoeae* strains (Recipients) with different ceftriaxone MICs and genetic resistance determinants affecting the susceptibility to ceftriaxone. The ceftriaxone MICs using the Etest method (shown as mean results of three repeated experiments) of the donor strain, recipient strains (R), and transformants (T) and the MIC ratio (T/R) are given. The breakpoint for ceftriaxone resistance is according to reference 8.

ceftriaxone resistance also and, although the biological fitness of ceftriaxone resistance in *N. gonorrhoeae* remains unknown, the gonococcus may soon become a true superbug that initiates a future era of untreatable gonorrhea. To at least slow the spread of ESC (cefixime and ceftriaxone) resistance, a reduction in global gonorrhea burden by enhanced disease prevention and control activities is crucial. As well, the implementation of much wider strategies for general AMR control, better understanding of the mechanisms and global monitoring of the emergence and spread of AMR, and global and national public health response plans (including sustainable clinical, microbiological, and epidemiological components) are needed. Any such plan alone will most probably not be able to prevent the emergence, establishment, and spread of ceftriaxone resistance; nevertheless, these plans will be valuable to delay and limit a global spread of ESC resistance (cefixime and ceftriaxone). Ultimately, a major focus important for public health globally is the timely development of effective new drugs (for single or combined use) for the treatment of gonorrhea.

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